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1 **Detection of neutralizing antibodies in postweaning multisystemic wasting syndrome (PMWS)-affected**
2 **and non-PMWS-affected pigs**

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26

27 **Abstract**

28 The notion that postweaning multisystemic wasting syndrome (PMWS)-affected pigs develop
29 an impaired humoral response against porcine circovirus type 2 (PCV2) has been reported in several
30 studies. However, little information is available regarding the presence of neutralizing antibodies
31 (NA) in PCV2-infected pigs and their role in the pathogenesis of the disease. The aim of the present
32 work was to further characterize the humoral response, and in particular the production of NA, in
33 pigs with different PCV2-infection status. Seventy-two conventional pigs from different farms were
34 classified into three groups based on PCV2 infection and clinico-pathological status, namely:
35 PCV2-negative, non-PMWS PCV2-positive and PMWS-affected animals. In addition, 9-week old
36 pigs from an experimental infection (6 controls and 14 PCV2-inoculated pigs) were also studied.
37 NA and total PCV2 antibodies (TA) as well as viral load in serum were determined and correlated
38 with the clinico-pathological status of pigs. Results indicated that PMWS-affected pigs had lower
39 NA titres, if any, than healthy animals. NA titres were also inversely correlated with PCV2 load in
40 serum. NA and TA titres were positively correlated; however, correlation differed among infection
41 status, being lower in PCV2-positive pigs. Also, the diagnostic performance of each test was
42 evaluated, indicating that the combination of viral neutralization and quantitative PCR in serum was
43 useful to discard PMWS (specificity 92%). In experimentally infected animals, the evolution of NA
44 paralleled the course TA, although a slight delay in NA production was seen in some animals. The
45 increase of NA coincided with the drop in viral load. Results from this work further support that
46 PMWS-affected pigs show an impaired humoral immune response and, particularly, an inefficient
47 NA response against PCV2.

48

49 *Keywords:* neutralizing antibodies; total antibodies; porcine circovirus type 2; postweaning
50 multisystemic wasting syndrome; diagnosis.

51

52 1. Introduction

53

54 The *Circoviridae* family comprises small, non-enveloped, icosahedral viruses with a circular,
55 single-stranded DNA genome that infect vertebrates. Two genera are included in this family:
56 Gyrovirus and Circovirus. Chicken anemia virus (CAV) is the only member in the genus Gyrovirus.
57 The genus Circovirus includes avian viruses, affecting canaries, psittacines, pigeons, ducks, ravens,
58 geese and starlings, as well as two swine viruses named porcine circovirus type 1 (PCV1) and type
59 2 (PCV2) (McNulty et al., 2000).

60 Among porcine circoviruses, only PCV2 is known to be pathogenic, being associated with
61 several diseases collectively designated as porcine circovirus diseases (PCVD) (Segalés et al.,
62 2005). PCVD include postweaning multisystemic wasting syndrome (PMWS), porcine dermatitis
63 and nephropathy syndrome (PNDS) and reproductive disorders. PCV2 has been also involved in the
64 porcine respiratory disease complex (PRDC). However, a clear causal association between PCV2
65 and the development of disease has only been demonstrated for PMWS and reproductive disorders
66 while its association with other diseases is still a matter of controversy.

67 PMWS is now recognized as a multifactorial disease for which the presence of the virus is a
68 necessary but not sufficient cause (Segalés and Domingo, 2002). Actually, most herds and pigs are
69 PCV2 seropositive but only a few suffer PMWS outbreaks or have individual PMWS cases
70 (Labarque et al., 2000; Rodriguez-Arriola et al., 2000; Segalés and Domingo, 2002). This
71 multicausal origin of PMWS is also supported by experimental data indicating the difficulty to
72 reproduce the syndrome by inoculation with PCV2 alone (Allan et al., 1999; Allan et al., 2000;
73 Balasch et al., 1999; Krakowka et al., 2000; Resendes et al., 2004). Much has been speculated on
74 the other causal factors needed for the development of PMWS and most studies point out at
75 immune-related factors (Darwich et al., 2004; Krakowka et al., 2001; Meerts et al., 2005).

76 Evidences that the host immune response is crucial in the pathogenesis of PMWS are

77 accumulating. PMWS-affected pigs have impaired cytokine responses (Darwich et al., 2003a;
78 Darwich et al., 2003b; Stevenson et al., 2006) both *in vitro* and *ex vivo* and it seems that secondary
79 or opportunistic infections are common in those animals (Segalés et al., 2004). Also, PCV2 may
80 modulate the activity of some dendritic cells (Vincent et al., 2005), and several studies reported that
81 PMWS-affected pigs show an apparently delayed antibody production (Bolin et al., 2001; Okuda et
82 al., 2003) or produce lower antibody titres to PCV2 compared to subclinically-infected ones
83 (Hasslung et al., 2005; Ladekjaer-Mikkelsen et al., 2002; Rovira et al., 2002). However, the role of
84 neutralizing antibodies (NA) is still poorly understood. Previous studies reported that NA against
85 PCV2 are produced during the course of the experimental and natural PCV2 infection (Meerts et al.,
86 2006; Meerts et al., 2005; Pogranichniy et al., 2000) and its absence has been associated with an
87 active viral replication leading to the development of PMWS (Meerts et al., 2006; Meerts et al.,
88 2005). However, these evidences came only from two experimental studies and two single farms
89 from Denmark and Belgium and thus it is difficult to figure out if this is a general phenomenon
90 taking place in the pig population.

91 The aim of this work was to compare NA titres among conventional pigs with different PCV2-
92 infection and clinico-pathological status, including field cases and experimentally PCV2-inoculated
93 pigs. Also, levels of NA were compared to total-PCV2 antibodies (TA) detected by an
94 immunoperoxidase monolayer assay (IPMA) and viral load in serum quantified by Real Time PCR.

95

96 **2. Materials and methods**

97

98 *2.1. Animals*

99 Field cases were randomly selected from the archival records (2002-2004) of the Diagnostic
100 Pathology Service of the Veterinary Faculty of Barcelona (Spain). Selected animals (n=72)
101 corresponded to pigs ranging from two to four months of age. Those animals came from 34

102 different farms located in North-eastern Spain. Animals were classified into two main groups
103 according to the presence of PCV2 genome in lymphoid tissues as detected by *in situ* hybridization
104 (ISH) (Table 1): PCV2-negative (n=9) and PCV2-positive (n=63). The later group was further
105 subdivided into two groups (non-PMWS and PMWS) using a set of widely accepted PMWS
106 diagnostic criteria (Segalés et al., 2005): (1) clinical signs compatible with the disease (weight loss
107 and wasting with occasionally dyspnoea and enlargement of inguinal lymph nodes), (2) presence of
108 characteristic histopathological lesions in lymphoid tissues (lymphoid depletion, histiocytic
109 infiltration and presence of inclusion bodies), and (3) presence of moderate to high amounts of
110 PCV2 within the lymphoid lesions. Pigs were classified in the PMWS group if the three above
111 mentioned criteria were fulfilled. Those pigs showing either clinical signs or some mild lesional
112 degree but only low levels of PCV2 in lymphoid tissues were classified into non-PMWS group. The
113 average age and standard deviation of each group was $2,88\pm 0,59$ for PMWS affected pigs,
114 $2,98\pm 0,67$ for PCV2 positive non-PMWS affected pigs and $3\pm 2,1$ for PCV2 negative pigs (non
115 significant).

116 Experimentally inoculated pigs were also included in the study. Animals came from a previous
117 work (Resendes et al., 2004) and corresponded to 14 PCV2-inoculated pigs and 6 uninoculated
118 controls from an experimental infection. In the aforementioned study, 9 week-old conventional pigs
119 were intranasally inoculated with 10^5 TCID₅₀ of PCV2 (PCV2-group) or mock inoculated with 2.5
120 ml of PBS (control group). Animals were clinically monitored and blood samples were taken at
121 weekly intervals, from 0 to 69 days post infection (dpi) to monitorize seroconversion (using IPMA)
122 and viraemia (by PCR and Real Time PCR). Serum samples from dpi 0, 7, 14, 21, 49 and 69 were
123 used to investigate the presence of NA. At the end of the study, tissue samples were collected for
124 histopathological analysis and detection of PCV2 genome by ISH. None of the PCV2-inoculated
125 pigs developed PMWS; however, serological and PCR results confirmed that animals had been
126 actually infected by PCV2. Controls remained free of the infection.

127

128 2.2. *Viral neutralization test (VNT)*

129 Several experiments were done in order to set up the test, including sample pre-treatment (heat
130 inactivated - 56°C, 30 minutes - versus non-inactivated samples), amount of virus particles to be
131 seeded in each well -100 TCID₅₀ versus 200 TCID₅₀ or 400 TCID₅₀-, and optimal incubation time of
132 inoculated cells (36, 48 and 72 h). The coefficient of variation of the neutralizing antibody titre intra
133 and inter test was also calculated.

134 Fifty microlitres of serum (inactivated and non-inactivated) tested were serially two-fold diluted
135 in 96-well plates, from 1:2 to 1:4,096 in complete DMEM (Dulbecco's Modified Eagle Medium
136 (DMEM), supplemented with 5% foetal bovine serum (FBS), 1% L-glutamine, 10,000 U/ml of
137 penicillin, 50 µg/ml streptomycin and 3% non-essential amino acids). From a PCV2 stock (Burgos
138 strain), produced as previously described (McNeilly et al., 2001) and adjusted in complete DMEM
139 to abovementioned concentrations, 50µl were added to each well. After one hour of mixture
140 incubation, 2×10^4 freshly trypsinised Swine Kidney (SK) cells were added to each well and
141 incubated for 36, 42 or 72 hours at 37 °C in 5% CO₂ atmosphere. Then, cells were washed twice
142 with PBS and fixed with cold absolute ethanol at -20 °C for 30 minutes. Plates were then incubated
143 for 1 hour at 37 °C with a hyperimmune serum against PCV2 diluted 1:200 in phosphate-buffered
144 saline containing 0.1% Tween 20 (PBS-Tween) and 1% bovine serum albumin. After washing with
145 PBS-Tween, peroxidase-labelled protein A ($0.6 \mu\text{g ml}^{-1}$) was added and plates were incubated for 1
146 hour at 37 °C. Finally, plates were washed with PBS-Tween and amino-ethyl-carbazole was added
147 to reveal the reaction. All samples were tested in duplicate. In each plate, a serum sample with a
148 known neutralizing antibody titre was included as a positive control, and complete DMEM was
149 used as negative control (one row of wells). Finally, the viral inoculum used in each VNT was
150 titrated to ascertain the accuracy of the viral concentration.

151 Plate reading was done under microscope at magnification of 100X. Three fields were examined

152 in each well. Cells with nuclear, cytoplasmatic or nuclear plus cytoplasmatic staining were recorded
153 as infected. The percentage of virus neutralization (%VN) at each dilution was calculated according
154 to the following formula: %VN= [1-(mean n° of positive cells of the two replicas of each serum
155 dilution/mean n° of positive cells in negative control wells)] x 100.

156 Three criteria were considered to validate the test: (1) the positive control should have a titre not
157 beyond one dilution above or below of the expected value, (2) the coefficient of variation of the 12
158 negative controls of each plate (calculated as the standard deviation over the average of labelled
159 cells in negative control wells) should be <20% and (3) the titration of the inoculum should yield no
160 less than 2×10^3 TCID₅₀ ml⁻¹ (100 TCID₅₀/well neither more than 10^4 TCID₅₀ ml⁻¹ (500
161 TCID₅₀/well).

162 Once a plate was validated, the NA titre was calculated as the reciprocal of the last dilution in
163 which a given serum sample was able to reduce by 50% or 90% the number of PCV2 infected cells.
164 These values were designated as VNT50 or VNT90. For most calculations, the VNT50 was used.

165

166 2.3. *Immunoperoxidase monolayer assay for detection of total PCV2-antibodies*

167 PCV2-specific antibodies were detected by IPMA based on a previously described protocol
168 (Rodriguez-Arrijoja et al., 2000). Serial four-fold dilutions of sera were done from 1:20 to 1:20480
169 on 96 well plates containing PCV2 infected and previously fixed SK cells. Plates were incubated for
170 one hour at 37°C and then were washed with PBS-Tween. Fifty µl of protein-A conjugated with
171 peroxidase (Sigma Aldrich P8651), at 0.6 µg/ml in PBS-Tween, were added to each well and
172 incubated for another hour at 37°C. Finally, plates were washed, and a substrate solution of 3-
173 amino-9-diethyl-carbazole in 0.1 M acetate buffer with 0.05% hydrogen peroxide was added to
174 reveal the reaction,

175

176

177 2.4. *Isotype-specific ELISAs for detecting PCV2-specific IgGs and IgMs*

178 PCV2-specific IgM and IgG antibodies were measured in experimentally infected pigs by using
179 a commercial ELISA test (Ingezim PCV IgG® and Ingezim PCV IgM®, Ingenasa, Madrid, Spain).
180 These tests are capture-ELISAs that use a recombinant PCV2 protein for coating the plates. The
181 ELISAs were performed according to the recommendations of the manufacturer. However, since
182 plates did not include uncoated wells, a modification was developed to increase accuracy of the
183 readings. Thus, for each serum, a corrected optical density (OD) was calculated as follows: $OD_c =$
184 $[OD_{\text{sample}} / (OD_{\text{positive control}} - OD_{\text{negative control}})]$.

185

186 2.5. *Polymerase chain reaction (PCR) and Real Time PCR*

187 PCV2 copies per ml of serum were quantified using a previously described Taqman PCR (Olvera et
188 al., 2004). Briefly, reactions were carried out in a 96-well plate including 4 ten-fold log dilutions of
189 PCV2 standard, both by triplicates. Moreover, a negative control (with autoclavated water as a
190 template) was added every four samples. Each reaction contained 900 nM of each primer, 150 nM
191 of PCV2 probe, 0.4 µl of IC kit, 12.5 µl of TaqMan Universal Master Mix (Applied Biosystems,
192 Foster City, CA, USA) and 2.5 µl of template. Autoclaved nanopure water was added to bring the
193 final volume to 25 µl. Amplification was carried out under Universal Cycling conditions (10 min at
194 95°C, 2 min at 50°C and 40 cycles of 15 s at 95°C, 1 min at 60°C). Amplification and quantification
195 was carried out in an ABIPRISM 7000, Sequence Detection System (Applied Biosystems, Foster
196 City, CA, USA).

197

198 2.6. *Sequence analysis of PCV2*

199 In order to ascertain if differences in the amino acid composition of different PCV2 strains
200 infecting pigs could account for differences in VNT results, a comparative analysis of the ORF2
201 sequences (cap protein) of the inoculum used in the VNT (Burgos strain) and the virus present in

202 some of the studied animals was done. Seven field cases from seven different farms, including four
203 non-PMWS and three PMWS-affected pigs and one animal from the experimental infection were
204 selected. The reference PCV2 strain, Stoon-1010 (kindly provided by Dr. Gordon Allan, Veterinary
205 Sciences Division, Department of Agriculture for Northern Ireland, Belfast, UK) was also analysed.
206 The cap gene was amplified and sequenced from nucleotide 998 to 1757 (PCV2-B genome;
207 GenBank Accession Number, AF112862) using specific primers (capFw 5' CTT TTT TAT CAC
208 TTC GTA ATG 3' and capRw 5' CGC ACT TCT TTC GTT TTC 3'). PCR was done using 2 mM
209 MgCl₂, 0.5 μM each primer, 0.2 mM of each dNTP and 0.03 U/μL taq polymerase. Cycling
210 conditions were as follow: 94°C 5 min, 35 cycles of 30 sec at 94°C, 1 min at 50°C and 1 min at
211 72°C, and a final extension cycle of 7 min at 72°C. Amplicon were sequenced using Big Dye
212 terminator 3.1 kit and ABIPrism 3700 automatic sequencer (Applied Biosystems, Forster City,
213 USA) following manufacturer recommendations. Nucleotide sequences were translated to the
214 predicted amino acids using Bioedit (Hall, 1998). Aligments were done using ClustalW (Thomson
215 et al., 1994).

216

217 2.7. *Statistical analysis*

218 Statsdirect and SPSS 14.0 were used for the statistical analysis. Mann-Whitney test and one-
219 way analysis of variance with Turkey-Kramer multiple comparisons were used to compare NA
220 titres, IPMA titres and viral load between groups. A cluster analysis was done using SPSS program
221 for NA titres and for viral load. The chi-square test was used to correlate the groups defined by the
222 clusters and clinico-pathological status. In a final step, the titres obtained in the VNT50, VNT90
223 and IPMA were used to construct a ROC curve with Statsdirect. For this, the PMWS group was
224 considered as diseased and the non-PMWS group as healthy. The optimum cut-off was calculated
225 by plotting the resulting sensitivity against 1-specificity for each possible titre. The level for
226 statistical significance was set at $p < 0.05$.

227

228 **3. Results**229 *3.1. Virus neutralization test optimisation*

230 No significant differences were observed comparing inactivated serum samples to non-
231 inactivated ones. However, in some samples, inactivation of the serum caused a slight decrease of
232 neutralising capacity (not more than one log). In regards viral inoculum, the use of 100 TCID₅₀/well,
233 yielded a too low number of positive cells to be counted and variability between replicates
234 increased. Best results were achieved using 200 and 400 TCID₅₀/well, but no differences were
235 observed between these amounts of inoculated virus. Finally, 48 and 72 h incubation of virus in cell
236 culture yielded same results, while those generated by 36 h incubation had lower reproducibility.
237 The coefficient of variation of the neutralizing antibody titre intra- and inter-test calculated was
238 below 12%. Therefore, the VNT was finally optimised by using non-inactivated serum samples, 200
239 TCID₅₀/well (4×10^3 TCID₅₀ ml⁻¹, 50 µl) and 72 h as incubation time of inoculated cells.

240

241 *3.2 Detection of NA in field cases*

242 All pigs in the PCV2-negative group and all non-PMWS PCV2-positive pigs had detectable NA
243 titres at VNT50. In contrast, in the PMWS group, 8 out of 29 (28%) did not show detectable
244 neutralizing antibodies in comparison to healthy ones ($p < 0.05$). Moreover, the average VNT50
245 titres of PMWS pigs (1:64) was lower than those of non-PMWS animals (1:256) or PCV2-negative
246 pigs (1:512) ($p < 0.05$). When results were evaluated using the 90% neutralization, the proportion of
247 positive pigs was also lower in PMWS group ($p < 0.01$) although values did not differ significantly
248 among PCV2-positive animals in different groups. To gain further insight on the possible origin of
249 these differences, a comparative analysis was done between VNT90 and VNT50 titres in PMWS
250 and non-PMWS pigs. Thus, PMWS pigs with negative results at VNT90 had a mean VNT50 titre of
251 1:4, while results of VNT90 negative non-PMWS pigs had a mean VNT50 titre of 1:16 ($p < 0.001$).

252 Similar differences were seen for VNT90 titres 1:4, that corresponded to a VNT50 titre of 1:128 in
253 non-PMWS and 1:32 in PMWS-affected pigs ($p<0.01$). When VNT90 titres higher than 1:16 were
254 compared, the corresponding VNT50 titres did not differ significantly between PMWS and non-
255 PMWS pigs. Results of the VNT are summarized in table 2.

256 Regarding the distribution of titres within a given group, PCV2-negative animals showed a high
257 variation of NA titres, ranging from very low (1:4) to very high titres (1:4,096). The correlation
258 between NA titres and the age of PCV2-negative pigs indicated that the older the animals, the
259 higher the titres ($R=0.90$; $CI_{95\%}$: 0.59-0.98, $p=0.01$) (Figure 1). This correlation between titres and
260 age was not found in any of the PCV2-positive groups.

261 Distribution of individual results on the VNT50 and VNT90 in PCV2-positive groups is shown
262 in Figure 2A. In this case, a high dispersion of results was observed in both sub-clinically infected
263 and PMWS-affected pigs. In order to figure out if NA titres allowed the discrimination of PMWS
264 and subclinically-infected pigs, a cluster analysis was done considering all PCV2-infected animals
265 as only one group. Using that statistical technique, two clusters were identified. The first one had its
266 centroid around a VNT50 of 1:16 and the other one was centred on 1:512. These two clusters were
267 correlated with the clinico-pathological status of the animal. Thus, using the later value (1:512) as a
268 cut-off, animals having NA titres $\geq 1:512$ were 2.35 ($CI_{95\%}$: 1.24-4.45) times more likely to be
269 subclinically infected than those with lower titres ($p<0.05$).

270

271 *3.3 Detection of TA in field cases*

272 Pigs suffering from PMWS had significant lower TA titres than those subclinically infected
273 ($10.3\pm 3.7 \log_2$ (titre) versus $12.5\pm 2.9 \log_2$ (titre); $p=0.01$). No significant differences were detected
274 between PCV2-negative and PCV2-positive groups.

275 A positive correlation between TA and NA to PCV2 was observed when results of all groups
276 were analysed together ($R=0.62$; $CI_{95\%}$: 0.45-0.75 $p<0.0001$). However, when data was analysed by

277 groups, the strength of correlation was different regarding to the infection status of pigs. Thus, for
278 PCV2-negative animals, correlation was high ($R=0.95$, $(CI_{95\%}: 0.76-0.99)$) while in PCV2-infected
279 animals was lower or almost inexistent ($R=0.43$ ($CI_{95\%}: 0.09-0.67$) for PMWS animals and $R=0.53$
280 ($CI_{95\%}: 0.17-0.74$) for non-PMWS pigs). In addition, plotting of IPMA and NA titres showed that
281 some PCV2-positive animals had high IPMA titres ranging from 1:1,280 to 1:20,480 concomitantly
282 with low (1:2 – 1:16) or absent NA at VNT50. These cases accounted for 17.8% of pigs in PMWS
283 group and 12.5% in the non-PMWS group ($p>0.05$).

284

285 *3.4 Detection of PCV2 DNA copies in serum of field cases*

286 The average number of DNA copies in serum was higher ($10^{8.5} \pm 10^{2.1}$) in the PMWS group than
287 in subclinically PCV2-infected pigs ($10^{5.5} \pm 10^{3.4}$) ($p<0.001$). Distribution of the individual results
288 within groups obtained in the Real Time PCR is shown in figure 2B. A cluster analysis considering
289 all PCV2-positive animals as one group showed the existence of two clusters. Thus, animals with
290 more than $10^{6.93}$ DNA copies ml^{-1} of serum had 2.05 more chances to suffer from PMWS
291 ($CI_{95\%}: 1.24-3.41$, $p=0.003$) than animals with lower viral loads. Correlation between NA titres and
292 results of the Real Time PCR were not statistically significant although a possible trend was
293 observed ($p=0.07$).

294

295 *3.5 Diagnostic performance of the VNT, IPMA and Real time PCR for the diagnosis of PMWS*

296 The results obtained in VNT and IPMA techniques were used in a ROC analysis to determine
297 their diagnostic performance for live animals and the optimal conditions in which those tests should
298 be applied. For VNT50, the optimal cut-off for PMWS diagnosis was a titre of 1:256. With this
299 value, the sensitivity of the test would be 0.588 ($CI_{95\%}: 0.407-0.753$) and the specificity 0.793
300 ($CI_{95\%}: 0.603- 0.920$). For the VNT90, the optimal cut-off was calculated to be a titre of 1:4. With
301 this value, sensitivity would be 0.536 ($CI_{95\%}: 0.339-0.705$) and specificity 0.710 ($CI_{95\%}: 0.520-$

302 0.860). For the IPMA, the optimal cut-off was a titre of 1:5,120 that yielded a sensitivity of 0.759
303 ($CI_{95\%}$:0.565-0.897) and a specificity of 0.656 ($CI_{95\%}$:0.547-0.814).

304 Since none of the tests alone was specific or sensitive enough to diagnose PMWS, a
305 combination of tests was further explored. Of all possible combinations, the serial use of Real Time
306 PCR and VNT50 yielded adequate results in terms of specificity. Thus, when a VNT50 \leq 1:256 and
307 a viral load by RT-PCR in serum $\geq 10^{6.93}$ were simultaneously considered to confirm or discard a
308 PMWS case, specificity was 0.920 ($CI_{95\%}$:0.793-0.985) and sensitivity was 0.655 ($CI_{95\%}$:0.470-
309 0.804). No other test combination improved these values of sensitivity and specificity.

310

311 *3.6 Detection of NA and TA in experimentally inoculated pigs*

312 All piglets were seropositive for both TA and NA at 0 dpi due to maternal immunity. From 0 to
313 14 dpi, NA and TA titres steadily declined in both groups and no statistical differences were seen
314 until 21 dpi. In the PCV2-inoculated group, seroconversion to PCV2 mostly occurred between 14
315 and 21 dpi and NA were usually detected at the same time than TA rise. However, some delay was
316 observed in four out of 14 PCV2-inoculated pigs, in which NA were detected later on. A positive
317 correlation was detected between NA and TA titres ($R= 0.85$; $CI_{95\%}$: 0.792-0.899, $p<0.001$). None
318 of the control animals seroconverted. Dynamics of PCV2-TA and NA production are shown in
319 Figure 3A and 3B, respectively.

320

321 *3.7 Detection of isotype-specific antibodies to PCV2 in experimentally inoculated pigs*

322 Development of anti-PCV2 IgMs occurred between 7 and 14 dpi, reaching a peak at 21 dpi and
323 then steadily declined until 49 dpi, when only 3 out of 14 animals were positive. Anti-PCV2 IgGs
324 appeared later than IgM, between 14 and 21 dpi, and titres increased until 69 dpi (Figure 4). Results
325 of the IgM and IgG ELISA were compared to the VNT50. This comparison showed a positive
326 correlation between anti-PCV2 IgG titres and NA titres ($R=0.76$; $CI_{95\%}$:0.64-0.85; $p<0.0001$), but

327 no correlation was observed between IgMs and NA titres.

328

329 *3.8 PCR and Real Time PCR in experimentally inoculated pigs*

330 PCV2 DNA was detected in sera of 12 out of 14 inoculated animals. Two pigs did not develop
331 detectable viremia although they seroconverted. Interestingly, those two pigs had also the highest
332 NA titre at 0 dpi (1:256). In PCR positive animals, viral DNA was firstly detected in sera between 7
333 and 14 dpi and then, viral genome was continuously or intermittently detected, depending on the
334 animal, until the end of the study.

335 Quantitation of the number of serum PCV2 copies indicated that most animals had a peak of
336 viremia between 14 and 21 dpi, experiencing then a decrease in viral loads until the end of the
337 experiment. Dynamics of viremia with regards to VNT50 is shown in Figure 5. The predominant
338 pattern was the appearance of TA and NA one or two weeks after the starting of viremia. The
339 increase in NA titres coincided with a drop in viral loads in serum. Pigs that had a delayed
340 production of NA with respect to TA, the drop of viral load was observed only after seroconversion
341 to NA occurred (data not shown).

342

343 *3.9 Sequence analysis of PCV2*

344 Theoretical amino acid sequences were obtained from the *cap* gene, aligned using ClustalW and
345 a neighbour-joining tree with 1000 bootstraps was performed. Four different sequences were
346 obtained, two among field samples and the two corresponding to Burgos and Stoon-1010 virus
347 isolates (sequences were considered different if at least one amino acid residue changed). When the
348 putative epitopes defined by Lekcharoensuck et al. (2004) were studied on the obtained sequences,
349 two groups of different epitopes were reported. The first group included the sequences obtained
350 from field cases, while sequences of Burgos and Stoon-1010 both belonged to the second group.
351 Therefore, the PCV2 inoculum used in the VNT (Burgos) differed from the virus present in the

352 serum of the pigs in some amino acids changes. Those changes were also observed in Stoon-1010
353 strain. Comparison among PCV2 obtained from field and experimentally infected animals included
354 in the analysis reported no differences in any of the studied epitopes.

355

356 **4. Discussion**

357

358 PCV2 is now accepted as the causative agent of PMWS but the mechanism by which PCV2
359 causes the disease is poorly understood and it remains unclear why only a small proportion of
360 infected pigs develops the disease. Recently, Meerts et al.(2005) showed a correlation between the
361 lack of neutralizing antibodies and an increase in the viral replication, and the same authors later
362 demonstrated an association between a poor NA response and the development of PMWS (Meerts
363 et al., 2006). However, further characterization of the role of NA was needed. Firstly, because there
364 is no standard procedure for PCV2 VNT and thus, results might vary if different protocols are
365 applied. Secondly, because the available data was limited to few sera and, finally, no evaluation of
366 the diagnostic performance of VNT had been done before. In the present study, it was shown that
367 PMWS-affected pigs lacked or developed low NA titres to PCV2, while the majority of subclinical
368 infected animals had significantly higher titres. Thus, the inability of some pigs to develop a strong
369 neutralizing antibody response could be interpreted either as a cause or consequence of the
370 development of the PMWS. Longitudinal follow-up studies including PMWS and non-PMWS
371 affected pigs would help in clarifying this point.

372 The difference in NA titres was more marked in the VNT50 than in VNT90, and thus, for low
373 NA responders, similar VNT90 titres in PMWS and non-PMWS pigs corresponded to significantly
374 different VNT50 titres. These results might have been due to different PCV2 strains present in pigs;
375 however, sequence analysis of PCV2 reported no differences in the putative epitopes among any of
376 the studied pigs. One possible hypothesis to explain this fact would be that anti-PCV2 antibodies

377 produced by PMWS-diseased pigs had less affinity for the virus than those produced by
378 subclinically infected ones, particularly in those pigs with low NA titres.

379 Within the PCV2-negative group, a high variation of NA titres was observed. This variation
380 correlated with the age of the pigs. The low NA titres detected in PCV2-negative pigs between 2
381 and 3 months of age could be the result of either the presence of remaining maternal immunity or
382 very recent seroconversion to PCV2; on the other hand, all PCV2-negative pigs showing high NA
383 titres came from fattening units (> 3 months of age), suggesting that those animals had been
384 previously infected and had been able to clear the infection.

385 Comparison among groups with regards to TA showed again that PMWS-affected pigs had
386 significantly lower titres than non-diseased animals; however, the strength of the correlation was
387 higher in PCV2 negative animals than in positive ones. Within the PCV2-infected animals, some
388 pigs had high titres at IPMA but low or absent NA. These observations might indicate either that
389 some pigs develop a humoral response lacking NA or that NA are developed later than non-NA. In a
390 previous study, Meerts et al. (2006) suggested that PMWS affected pigs may be unable to produce
391 antibodies against certain neutralizing epitopes. This hypothesis was based on observations made
392 under experimental and field conditions in which PMWS pigs lacked NA but developed TA titres
393 similar to subclinically infected animals. However, in our case the lack of NA in presence of high
394 TA titres was only observed in a small proportion of PMWS pigs. Thus, from our results it seems
395 that the impairment of the humoral response may affect both NA and non-NA.

396 In the present study, and in accordance with others (Ladekjaer-Mikkelsen et al., 2002; Liu et al.,
397 2000; Olvera et al., 2004), PMWS was also related to high viral load in serum. We could not
398 demonstrate an inverse association between NA titres and PCV2 load in serum, although p-value
399 was close to significance ($p=0.07$). These results reinforce the notion that NA are essential to cope
400 with viral dissemination through blood. This lack of significance might be attributable to the fact
401 that Real Time PCR detects viral DNA, either free or bound to antibodies, while NA does not detect

402 antibodies already coupled to virus. In addition, coexistence of NA with viral DNA was observed in
403 some of the animals, both affected and non-affected by PMWS, suggesting that NA alone might not
404 be enough to produce the viral clearance. Viral persistence despite high levels of NA was also
405 observed in the experimentally PCV2-infected pigs and has been also reported for chicken anaemia
406 virus, a related virus of the *Circoviridae* family (Brentano et al., 2005).

407 In this study, we also included an evaluation of the diagnostic performance of VNT, IPMA and
408 Real Time PCR for the diagnosis of PMWS. This is relevant because all these tests can be
409 performed on live pigs and may be helpful to establish a diagnosis on a population basis. The use of
410 ROC curves showed that VNT50 yielded the best specificity (0.793) and IPMA had the highest
411 sensitivity (0.759). Nevertheless, both tests showed serious deficiencies in sensitivity (VNT50) or
412 specificity (IPMA). However, the combination of Real Time PCR and VNT50 yielded an acceptable
413 specificity (0.92) and could be used to potentially discard PMWS in live animals. One important
414 consideration to be made is that the current definition of PMWS implies the need of a
415 histopathological analysis with grading of lesions and amount of virus in tissues, which both require
416 certain expertise. Therefore, different laboratories might give different interpretations to the same
417 case. This is not crucial in evident PMWS cases but produces a grey zone of intermediate (slight to
418 moderate lesions and low to moderate amount of PCV2) interpretations. As a result, diagnosis
419 would be difficult in these cases, and tests such as the VNT or the Real Time PCR may help to
420 refine the diagnosis.

421 VNT results obtained for the experimentally inoculated pigs confirmed that animals develop NA
422 during the course of a subclinical infection. All pigs from both control and PCV2-inoculated groups
423 had maternal-derived NA. The neutralizing capacity of maternal-derived antibodies had been only
424 reported once in naturally PCV2-infected pigs (Meerts et al., 2006). In that study, no significant
425 differences were observed in NA titres among subclinically and PMWS-affected animals. In our
426 case, two of the PCV2-inoculated pigs did not developed viremia. Those animals had the highest

427 NA titre at day 0 (1:256) and this is suggestive that, most probably, if maternal antibodies have a
428 protective role against PCV2, that depends on the NA titre attained. This notion would agree with
429 other studies (McKeown et al., 2005; Ostanello et al., 2005), where maternal-derived protection
430 seemed to be dependent on the total antibody titres.

431 The neutralizing capacity of antibodies seems to be mainly restricted to IgGs but not IgMs as
432 shown by the correlation between isotype-specific ELISAs and NA results. In addition, PCV2-
433 specific IgMs seem to be short-lived and decreased below the detection limit 2-3 weeks after
434 seroconversion. These observations differ from those of Meerts *et al.* (2006), in which IgM
435 persistence was observed in subclinical PCV2-infected pigs up to six weeks after seroconversion.
436 The reason for such a discrepancy is unknown to us but might lie in a different sensitivity of the
437 tests used.

438 The drop of the viremia most often occurred simultaneously with an increase in the NA titre, as
439 seen from the serological and Real Time PCR profiles in PCV2-inoculated animals. Actually, in
440 those animals with a slight delayed NA response in comparison to the appearance of TA, viremia
441 dropped only after the pig seroconverted for NA. These observations suggested that viral circulation
442 in blood is reduced by an antibody-mediated neutralization, being presumably an important
443 mechanism in the viral clearance and recovery of the infection.

444 In summary, the present study confirms that the lack of NA is related to PMWS. In addition, we
445 have also shown that evolution of viremia and development of NA are inversely related and that the
446 combination of VNT and Real Time PCR may be useful as a diagnostic criterion for excluding non-
447 PMWS cases. Nevertheless, further studies are needed to elucidate the mechanisms lying under the
448 apparent inability of some pigs to develop a full humoral response with NA as well as on their
449 contribution to the immunopathogenesis of PMWS.

450

451

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453

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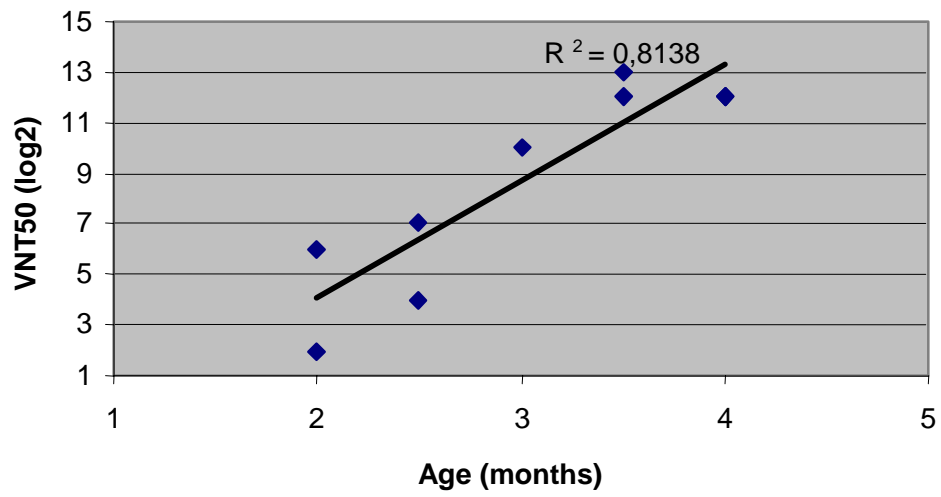
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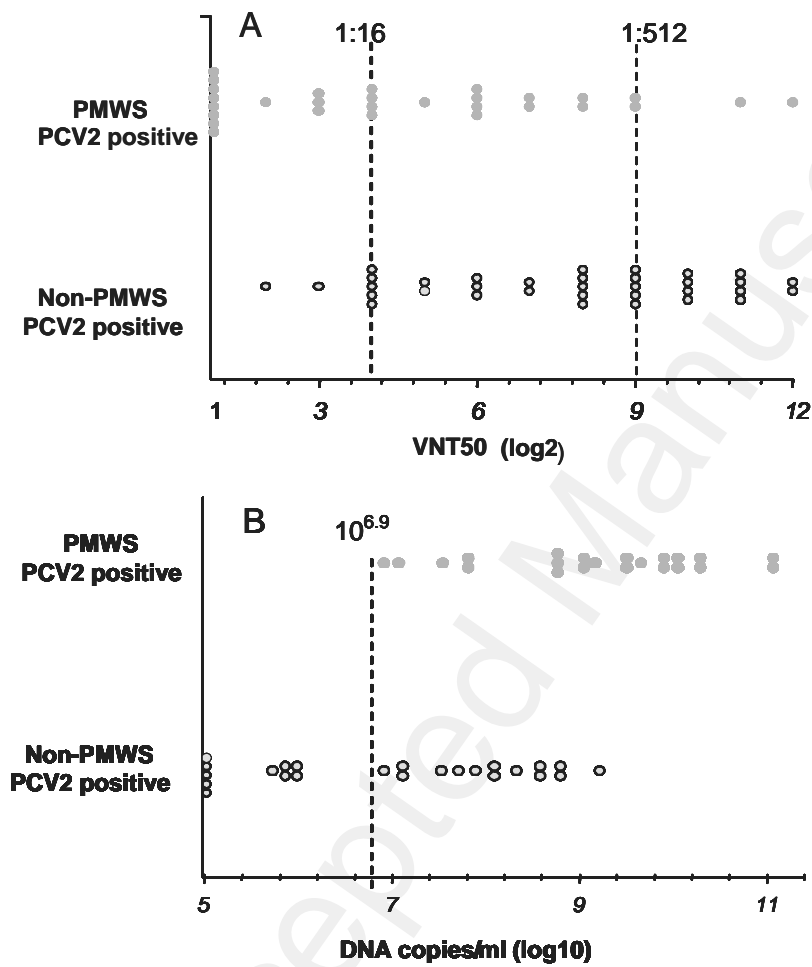
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- 580

- 1 **Fig. 1.** Correlation between NA titre and age of pigs in PCV2-negative group. VN =
2 virus neutralization.

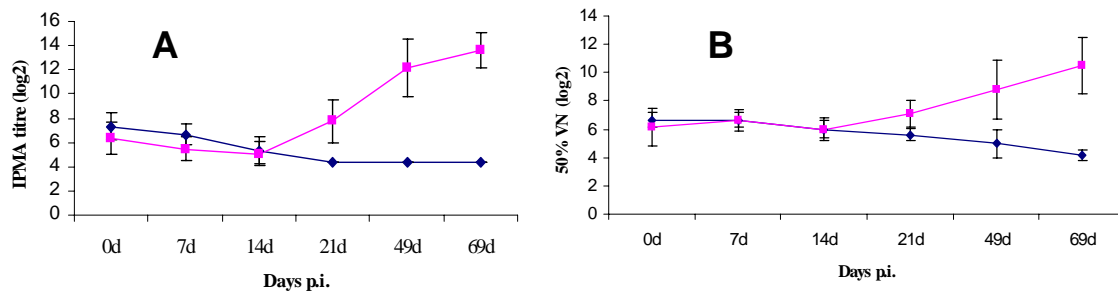


- 1 **Fig. 2.** Distribution of NA titres at VNT50 (A) and number of DNA copies/ml of serum
2 (B) in PMWS and non-PMWS PCV2-positive groups. Centroids of clusters (A) and the
3 calculated threshold between clusters (B) are indicated by dashed lines.
4



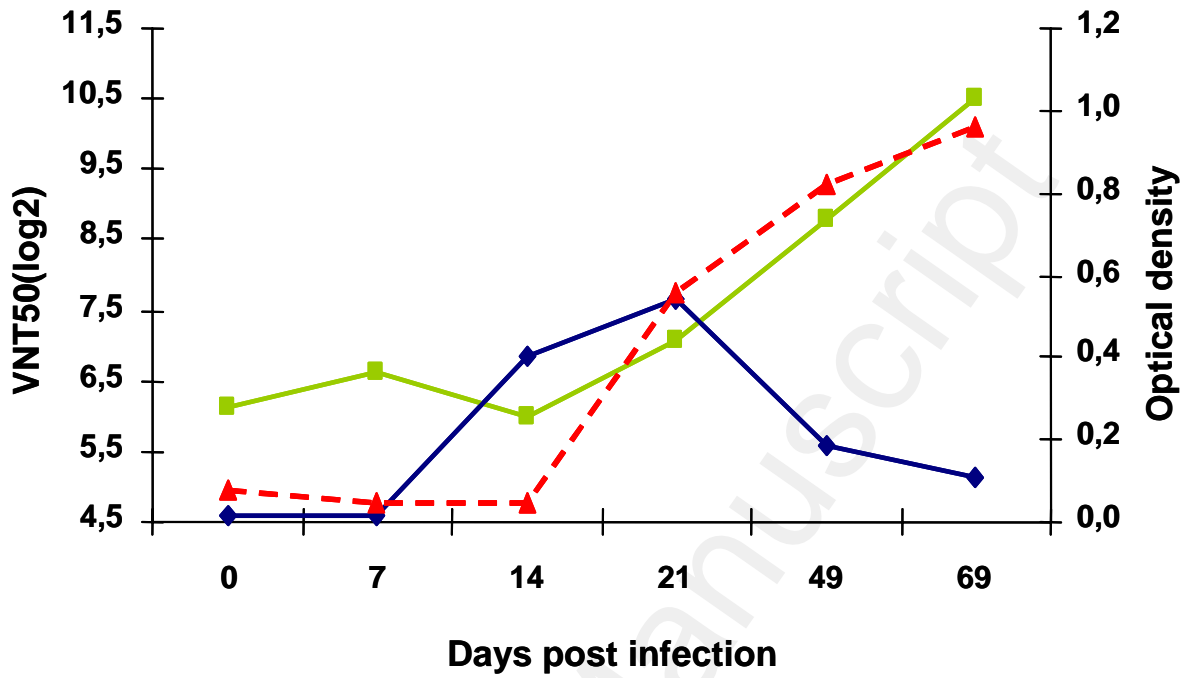
1 **Fig. 3.** Serological evolution in controls (—◆—) and PCV2-inoculated pigs (—■—) from
2 0 to 69 dpi. **A)** Average TA titres against PCV2 **B)** Average NA titres (VNT50) to
3 PCV2.

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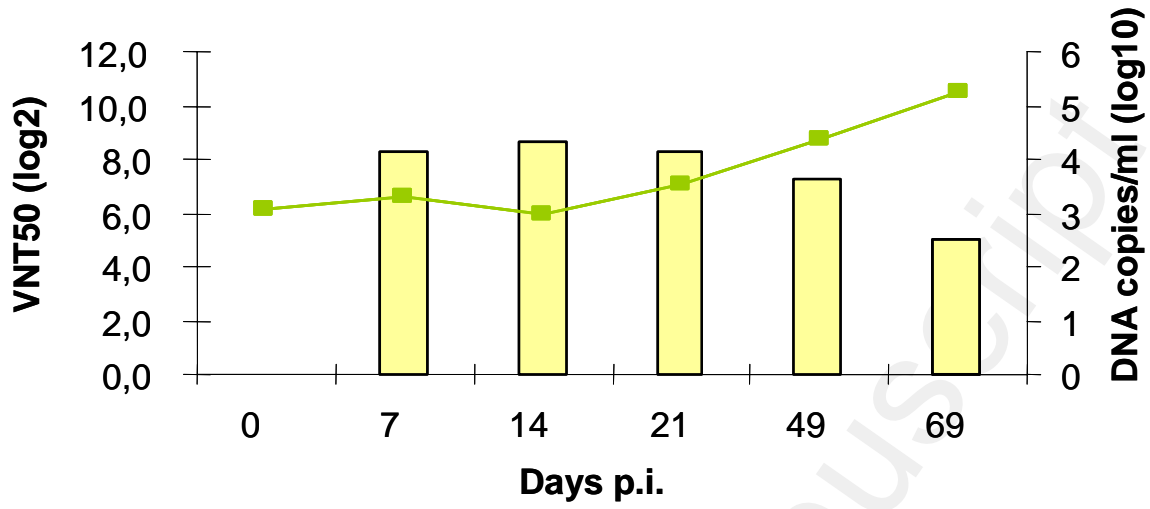


1 **Fig. 4.** Mean NA titres (VNT50) (—■—) and mean optical densities of anti-PCV2 IgGs
2 (---▲---) and IgMs (—◆—) in PCV2-inoculated pigs (0 to 69 days post infection).

3



1 **Fig. 5.** Dynamics of NA (—■—) and viral loads (□) in PCV2-inoculated pigs.



1 **Table 1.** Groups of pigs included in the study according to the PCV2-infection and
 2 clinico-pathological status.

3
 4
 5

PCV2 status	Group	<i>In situ</i> hibridisation		Histopathological lesions	
		Presence of PCV2 genome	Amount of PCV2 genome	Presence of lesions	Severity of lesions
Negative	I	No	N.A.*	No	N.A.
Positive					
Non-PMWS	II	Yes	Low	Yes/No	Slight/Nil
PMWS	III	Yes	Moderate to high	Yes	Moderate to severe

6 * Not applicable

7

1 **Table 2.** Percentages of pigs with neutralizing antibodies and mean NA titres at both
 2 50% and 90% of viral neutralization end-point.

3

4

	VNT50		VNT90	
	% of pigs	Mean titre of positives	% of pigs	Mean titre of positives
PCV2 -ve	100 (9/9)	1:512	77,8 (7/9)	1:16
PCV2 +ve				
Non-PMWS	100 (34/34)	1:256	71 (22/31)	1:16
PMWS	72,4 (21/29)	1:64	46,4 (13/28)	1:8

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